AMENDMENT

In the Specification:

Please amend the first paragraph of the specification entitled "Cross-Reference to Related Applications" as follows:

This application ~~claims priority to~~ is a divisional of U.S. patent application Serial No. 09/697,022, filed 25 October, 2000, now U.S. Patent No. 6,627,427, issued 30 September 2003, which claims the benefit of the filing date of 60/161,414, filed 25 [[Oct.]] October 1999~~, and is related to U.S. patent application Serial Nos. 60/161,703, filed 27 Oct. 1999, and 60/206,082, filed 18 May 2000,~~ each of which is incorporated herein by reference.

Please insert the following section below the section entitled "Cross-Reference to Related Applications" and above the section entitled "Field of the Invention":

Reference to Government Funding

This invention was supported in part by Grant No. AI51106. The U.S. government has certain rights in this invention.

Please insert the following text at page 5, line 26, of the specification:

Figures 3 A-C show the *eryM* gene sequence with the engineered *Xba*I and native *Hin*dIII sites (SEQ ID NO: 1 and SEQ ID NO:2).

Figure 4 shows production of 15-methyl erythromycin A and B by a wild type Saccharopolyspora erythraea strain and two isolates of the Saccharopolyspora erythraea eryM$^-$ mutant as described in Example 1.

Figure 5 shows the pathway and genes for erythromycin biosynthesis in *Saccharopolyspora erythraea*.

Figures 6A-6C show pathways for the synthesis of acyl-CoA precursors in *Streptomyces*.

Figure 7 shows the erythromycin PKS and synthesis of 6-deoxyerythronolide B.

Figure 8 shows the pathways to TDP-desosamine and TDP-mycaminose.

Figure 9 shows production of a 10,11-alkene from 15-methylerythromycin A.

Figures 10 A-C show the amino acid sequence of a hybrid PKS (SEQ ID NO:3).

Figures 11 A-B show the amino acid sequence of another hybrid PKS (SEQ ID NO:4).

Figures 12 A-B show the amino acid sequence of a final hybrid PKS SEQ ID NO:5).

Please replace the paragraph beginning on page 38, line 18 with the following paragraph:

The *Saccharopolyspora erythraea eryM* gene was isolated by PCR of the coding region. The cloning vectors pWHM3 and pOJ260 are well-known Streptomyces vectors. An internal fragment of the *eryM* gene was isolated by PCR and cloned into the *Xba*I and *Hin*dIII sites of the vectors pWHM3 (confers thiostrepton resistance) and pOJ260 (confers apramycin resistance) for gene disruption. The resulting vectors were propagated in *E. coli* ET12567 to obtain unmethylated DNA. The *eryM* gene sequence showing the engineered *Xba*I and native *Hin*dIII sites used to clone the internal fragment into the vectors is shown in Figures 3 A-C [[below]]. The *Xba*I site introduces a stop codon into the reading frame, ensuring that insertion by homologous recombination will disrupt the gene.

Please delete the gene sequence which begins on page 38, line 27, and ends on page 40, line 45, as this gene sequence is now represented by Figures 3 A-C.

Please replace the paragraph beginning on page 41, line 15 with the following paragraph:

The parent strain and two isolates of the *eryM*⁻ mutant were grown using the shake flask procedure. In addition to the oil plus propanol feed, culture flasks were fed equivalent levels of oil alone, oil plus butanol, oil plus propionate, and oil plus butyrate. The cultures were killed by the propionate and butyrate feeds, and these flasks were discarded. Samples were taken from the other flasks each day and the set was analyzed by ion counting. The results are shown graphically below. The first graph shows a time-course of production of erythromycins A and B for the wild-type and

mutant strains with the different feeding regimes. The second graph shows the same for 15-methyl-erythromycins A and B (Figure 4). The ion count at 748.6 amu is not all due to 15-methyl-erythromycin A. LC-MS analysis of ethyl acetate extracts of day 7 samples fed oil alone or oil and butanol suggested that only about 10% of the 748.6 peak was 15-methyl-erythromycin A. The exact amount of PrEryA produced by the strains remains to be determined.

Please delete the Figure provided on page 44 as this Figure is now provided as Figure 4.

Please replace the paragraph beginning on page 45, line 5 with the following paragraph:

While Example 1 illustrates the aspect of the invention in which butyryl CoA is loaded by the loading domain of DEBS to produce 15-methyl-6-deoxyerythronolide B, this and the following Example illustrate an alternative aspect of the invention, in which a recombinant PKS that comprises an altered loading domain is used to produce the compound. This altered loading domain can be employed with any number of extender modules from any one or more PKS. In a preferred embodiment, the loading domain is used in conjunction with the six extender modules of DEBS, or the oleandolide PKS (see PCT patent publication No.WO 00/026349, incorporated herein by reference), or the megalomicin PKS (see U.S. patent application Serial No. 60/190,024, filed 17 Mar. 2000, and the application Serial No. 09/679,279 ~~_____, attorney Morrison and Foerster docket no. 30062-20047.20~~, filed 4 Oct. 2000, naming the same inventors and claiming priority to the former applicaton, each of which is incorporated herein by reference), to produce 15-methyl-erythromycins in *Saccharopolyspora erythraea* host cells.

Please replace the paragraph beginning on page 45, line 18 with the following paragraph:

An illustrative hybrid PKS of the invention is made by replacing the AT domain of the loading module of the oleandomycin PKS with the ethylmalonyl-CoA specifying AT domain of the fourth extender module of the FK520 PKS. The resulting hybrid PKS contains the KSQ domain and downstream interdomain region of OleA1 (aa 1-562) fused to the FKAT4 domain (aa 562-896) fused to the OleA1 AT-ACP interdomain region, adjoining OleA1 ACP of the loading domain and the remainder of the OlePKS (897 - end). The amino acid sequence of the hybrid portion of this PKS is shown in Figures 10 A-C **[[below]]**.

Please delete the gene sequence which begins on page 45, line 26, and ends on page 46, line 25, as this gene sequence is now represented by Figures 10 A-C.

Please replace the paragraph beginning on page 46, line 26 with the following paragraph:

Another illustrative hybrid PKS of the invention is made by fusing the following in order specified: the first 9 aa of OleAI (1-9 in sequence below); 846 aa of the FK520 PKS encompassing the KS and AT domains of module 4 as well as the KS-AT interdomain region (10-855); the ATL - ACPL interdomain region of OleAI, followed by the ACPL domain and the rest of the OLE PKS (856 - end). The amino acid sequence of the hybrid portion of this PKS is shown in Figures 11A-B [[below]].

Please delete the gene sequence which begins on page 46, line 34, and ends on page 47, line 21, as this gene sequence is now represented by Figures 11 A-B.

Please replace the paragraph beginning on page 47, line 23 with the following paragraph:

The hybrid PKS above is then changed in the DNA sequence corresponding to aa 177 so that the C is replaced by a Q residue in the final hybrid PKS, yielding the ~~following~~ amino acid sequence of Figures 12 A-B.

Please delete the gene sequence which begins on page 47, line 27, and ends on page 48, line 13, as this gene sequence is now represented by Figures 12 A-B.

Please delete the figure starting on page 53 at line 1 and continuing until line 3, as this figure is now represented by Figure 5.

Please replace the sentence beginning on page 53, line 3 with the following sentence:

Figure 5 shows the [[P]]pathway and genes for erythromycin biosynthesis in *Sac. erythraea*.

Please replace the paragraph beginning on page 55, line 9 with the following paragraph:

Several other routes to mm-CoA are shown below. One is the conversion of succinyl-CoA to mm-CoA via the enzyme methylmalonyl CoA mutase, which has been identified *in S. cinnamonensis*, producer of the polyketide monensin (20). Succinyl-CoA arises from the oxidation of acetyl-CoA via the Krebs cycle, but requires the input of oxaloacetate, which would be derived from the breakdown of glucose. Because the utilization of glucose is generally controlled in high-titer fermentations, and because it serves as the principal source for the synthesis of the deoxysugars present in macrolide antibiotics, it is unlikely that it serves as a significant source of precursors. Succinyl-CoA could also come from alphaketoglutarate, produced from deamination of glutamate. Fermentations fed with succinate or its purported precursors have only shown marginal increases in titers of polyketides, indicating that the succinyl-CoA pathway is not a major contributor to the large precursor pools required for high level synthesis, but this has not been ruled out in the high titer strains. [[The f]]Figures 6A-C [[below]] shows pathways for the synthesis of acyl-CoA precursors in *Streptomyces*. Enzymes are shown in bold: VDH, valine dehydrogenase; LDH, leucine dehydrogenase; MMCoA mutase, methylmalonyl CoA mutase.

Please delete the Figure provided on page 56 as this Figure is now provided as Figures 6A-C.

Please replace the paragraph beginning on page 59, line 4 with the following paragraph:

Heterologous expression of DEBS in *S. coelicolor* (45) yielded a mixture of 6-dEB and 8,8a-deoxyoleandolide, at levels up to 50 mg/L[[.]] DEBS can thus initiate polyketide synthesis *in vivo* either from p-CoA or acetyl-CoA. Similarly, the DEBS1 protein (containing the loading module and modules 1 and 2) was re-engineered by placing the TE domain after the ACP domain of module 2. In *S. coelicolor,* the DEBS1-TE construct yielded the predicted triketide products 2,4-dimethyl-3,5-dihydroxyheptanoic acid delta-lactone [**14**] and 2,4,-dimethyl-3,5-dihydroxyhexanoic acid-delta-lactone [**15**] shown below (46). It is thus not always necessary to replace the DEBS loading domain to alter the starter unit. Figure 1 and Figure 7 ~~the schematic below~~ show the erythromycin PKS and synthesis of 6-deoxyerythronolide B.

Please remove the schematic provided on page 59, starting at line 15, as this schematic is now provided as Figure 7.

Please replace the sentence beginning on page 61, line 4 with the following sentence:

~~The schematic below~~ Figure 8 shows the pathways to TDP-desosamine and TDP-mycaminose.

Please remove the schematic provided on page 61 as this schematic is now provided as Figure 8.

Please replace the paragraph beginning on page 62, line 6 with the following paragraph:

The initial transformation of 15-methylerythromycin A into the 6-*O*-methyl derivative follows standard procedures (52) and proceeds in good overall yield. The key intermediate in the synthetic process is the 10,11-alkene [**18**], which is produced similarly to reported ketolides (53). The 6-*O*-methyl analog is subjected to acid-mediated removal of the cladinose, and the resulting 3-hydroxy group is oxidized to the ketone after protection of the desosamine 2'-hydroxyl as the acetate. The 11-hydroxyl group is converted to the mesylate, then eliminated by treatment with diazabicycloundecene (DBU) to introduce a 10,11-alkene functionality. This procedure results in an overall 10-15% yield of **18** starting from the initial 15-methylerythromycin A (see Figure 9).

Please remove the schematic provided on page 63, lines 5 to 7, as this schematic is now provided as Figure 9.

Please replace the paragraph beginning on page 70, line 20 with the following paragraph:

As described above, there are several routes to the synthesis of the various acyl-CoAs required for polyketide biosynthesis (illustrated in Figures 6A-C ~~the schematic above~~). Because the valine utilzation pathway represents a major route to the required precursors, one can shunt some of the pathway intermediate ib-CoA to b-CoA. First, one can check the levels of b-CoA in fermentations fed with high valine content proteinaceous substrates to establish a baseline. Then, one can introduce the *S. cinnamonensis* genes *icmA* and *icmB* for ib CoA mutase into the *Sac. erythraea* host under the control of the *ermE** promoter and examine the cells for the amount of 15-MeEryA and EryA produced. One can also examine cells for the levels of b-CoA and ib-CoA. The degree of success of this method depends on the host either containing a large pool of ib-CoA or

there being sufficient flux through the valine degradation pathway to provide enough ib-CoA to be converted efficiently to the required b-CoA to promote its high level incorporation in polyketide synthesis.

Please replace the paragraph beginning on page 71, line 5 with the following paragraph:

If one does not see the desired effect of the *icmA* and *B* genes on 15-MeEryA or b-CoA levels in a cell and the cells contain ib-CoA mutase activity, particularly in cultures fed with high valine containing proteins, one can examine pathway flux by measuring the level of BKD activity in erythromycin-producing *Sac. erythraea* cultures (see Figure 6A ~~the schematic above~~). If one finds indistinguishable levels of activity in low and high erythromycin-producing strains, one can in accordance with the methods of the invention overexpress the *S. coelicolor bkdF, G* and *H* genes, which have been identified in the *S. coelicolor* genome, in the appropriate *Sac. erythraea* host containing *icmA* and *icmB* and determine the effect on 15-MeEryA and b-CoA levels.